Single molecule labeling of an atomic force microscope cantilever tip

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In this paper, we present a method to functionalize the very apex of an atomic force microscope cantilever with a single or a few molecules. In force spectroscopy or interaction mapping, the cantilever must be functionalized with only a few molecules to avoid noise or spurious measurements. Here, we covalently attached single molecules to the cantilever by touching it to a paper wetted with a solution of quantum dots. The paper competes with wicking up the hydrophilic surface of the tip. This method has broad applications in scanning probe microscopy where small numbers of molecules are needed on the tip. © *2012 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4760283]

Atomic force microscopy (AFM) can be used to measure surfaces of material samples at nanometer resolutions. In addition, when the AFM probe is labeled with molecules, it can be used as a "molecular sensor." Such a labeled AFM probe can not only measure the interaction force between molecules (dynamic force spectroscopy)[1] but also map the location of receptors on a cell membrane surface (interaction mapping or affinity mapping).[2] To measure these interactions and positions accurately, the cantilever tip should ideally be labeled with only a single or a few ligand molecules. However, existing methods to label the AFM tip, which simply submerge the tip into a droplet of molecules or let molecules in gas phase deposit on the probe, result in the labeling of large numbers of molecules onto the probe. These excess molecules create noisy data for force spectroscopy or generate spurious signals in interaction maps. Here, we report a technique to label AFM probes with a single ligand molecule by touching the tip to a piece of paper wetted with the molecules to be labeled. The fibers of the paper trap the labeling solution, but gentle pressure by the cantilever tip releases a small amount of solution and allows coating of the tip. The wicking action of the paper prevents the wetting of a large portion of the tip. In this report, we demonstrate the method by capturing fluorescent quantum dots (QDs, Life Technologies) on the tip, so that we can quantify the labeling by these molecules using fluorescence microscopy.

To label the tip, our design strategy was to use amines covalently attached to the tip as a tether for molecular attachment. The spring constant of the tip (SHOCONG, AppNano) was around 200 pN/nm calibrated using the thermal noise method.[3] The tip (SHOCONG, AppNano) was cleaned in a plasma cleaner, and then coated with the amino-silane APTES (Fisher Scientific) by vapor deposition (Fig. 1(a)).[4] We reacted carboxylic acid-modified QDs with EDC (Thermo Scientific), so that they could be cross-linked to the amines on the cantilever (Fig. 1(b)). We put a piece of common index paper (Staples, 30% recycled 20 lb paper) on a coverslip on the sample stage and pipetted ~10 μL MES buffer (100 mM, *p*H 5) to wet the paper. We then added 5 μL of the QD-EDC solution (5 μM) to the center of the wet region.

Existing methods of coating the tip involve dipping or submerging the tip in a labeling solution (Fig. 2(a)),[5] which because of wicking results in broad labeling across the tip (Fig. 2(b)). To label only the apex of the tip, we introduced the method of touching the tip to a wetted piece of paper, which bears the molecules to be labeled (Fig. 2(c)). One key step in this method is to ensure that the AFM tip only barely touches the paper. Any more than minimal contact allows the solvent to wick up the hydrophilic surface of the cantilever tip. We achieved this goal by iteratively lowering the AFM head (MFP 3D-BIO, Asylum Research) while the *z*-piezo ran a series of force-curves, extending and retracting the tip using a low force trigger (200–500 pN). By using such a low force trigger, we were assured the tip would stop extending downward into the paper as soon as it gently hit the wet paper.

After we established this distance from the AFM head to the paper, we changed the operating mode of the AFM to make the cantilever dwell on the wet paper upon contact. We

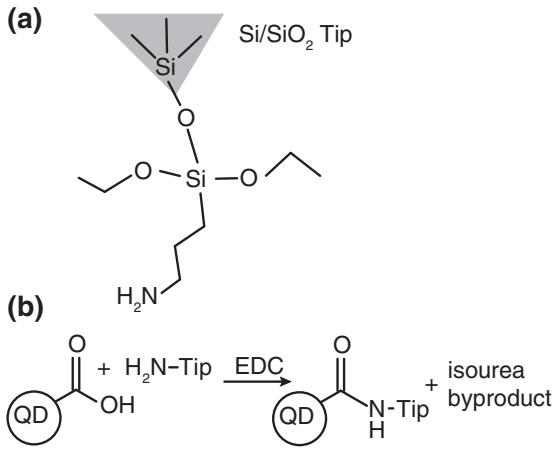

FIG. 1. Cross-linking strategy. (a) Presumed model of APTES binding to the silicon surface of AFM tip through the reaction between silanol groups on the tip and one or more alkoxy groups of APTES. (b) EDC reacts with the carboxylic acid group of the QD, which provides an activated leaving group in the reaction with the amine moiety of APTES.

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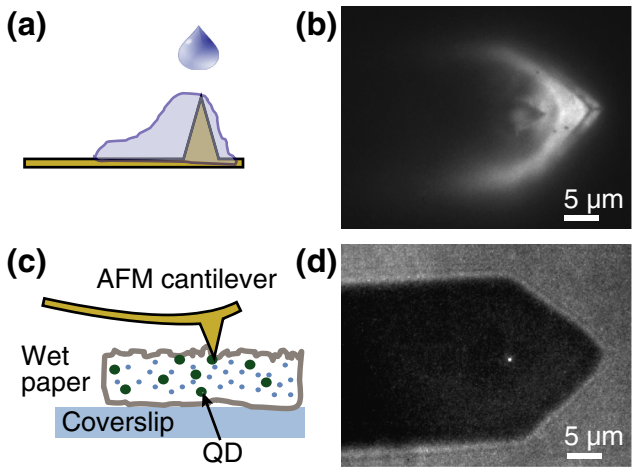

FIG. 2. (a) A common way to label AFM tip is to immerse the tip or to dip the tip into a small droplet. (b) Fluorescent widefield image (e.g., 405 nm excitation, emission filter 525/50) of AFM tip labeled using conventional labeling method shows QD525 molecules all over the tip. (c) The method of this paper entails touching the tip gently and repeatedly to wetted paper. (d) Fluorescent widefield image of AFM tip labeled using our method shows QD525 molecules are confined to the very end of the tip (bright spot).

found the deflection decreased gradually during the dwell (Fig. 3(a)), showing that the tip was pulled downward into the paper, due to wetting of the hydrophilic surface of the cantilever tip; we saw no such pull with dry paper or unsilanized tips. Too much pulling would result in too much surface area of the tip being labeled, yet the EDC chemistry requires some non-trivial contact time. We addressed this tradeoff by performing ten cycles of 10 s dwells, separated by full retractions of the *z*-piezo. The dwell periods provided 100 s of contact time for the cross-linking chemistry to occur. We observed a strong adhesion force when the tip was pulled out of contact with the wet paper (Figs. 3(a) and 3(b)). We hypothesize this adhesion force was due to interaction between hydrophilic silanized tip and water in the paper. As some QD may have stuck to the tip non-specifically (i.e., non-covalently), we removed non-covalently bound QD by immersing the tip in PBS buffer for 30 min.

The fluorescence image of tips labeled by our method shows a very small region of fluorescence localized at the tip

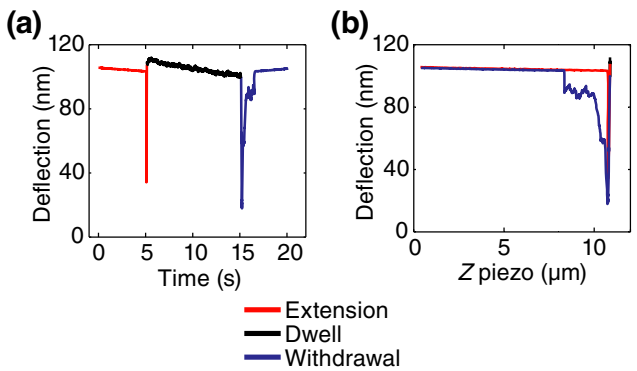

FIG. 3. (a) The deflection signal decreases gradually during the 10 s dwell (black) indicating the AFM cantilever was pulled downwards into the wet paper. A quick "jump to surface" (red) is seen just before the point of contact. (b) A large adhesion force is seen between tip and wet paper upon withdrawing the tip (blue, also seen in (a)). (a) and (b) show the same force curve.

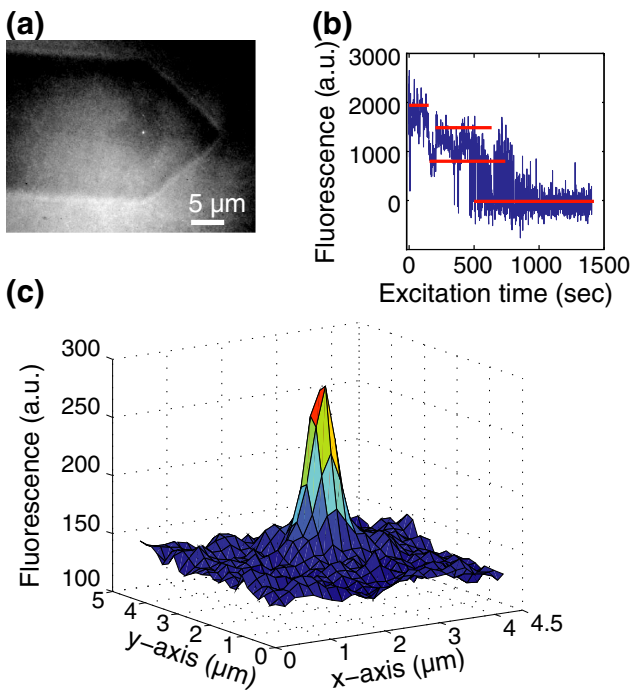

FIG. 4. (a) Fluorescence image of a labeled tip, showing only the very apex bears QDs. (b) Trajectory of background-corrected fluorescence intensity vs. time under continuous excitation. The presence of steps indicates that very likely about 3 QDs are labeled to the tip. (c) Fluorescence intensity of the same tip as (a), showing that QD(s) on the tip lie within a diffraction limited pot.

(Fig. 4(a)). The FWHM of this spot is 450 nm (Fig. 4(c)), very close to the diffraction limit (430 nm). By comparison, measurement of single QDs on glass showed a FWHM fluorescence of 440 nm. This result indicates that the labeled QD on the tip is localized to an area as small as a single QD could be resolved.

We estimated the number of labeled QD by photobleaching.[6] By measuring the intensity change as the fluorophores bleach, we obtained the fluorescence intensity of a single QD, and, thus, calculated the number of QD on the tip. A 405 nm diode laser (CUBE 405–100c, Coherent) was used to excite the QD. The fluorescence was measured on an inverted microscope (Eclipse Ti-E, Nikon) using a 60x objective (Apo TIRF NA 1.49, Nikon) and imaged by an intensified CCD camera (XR/MEGA-10, Stanford Photonics). To measure the photobleaching of the QD, the tip was excited by the laser without stopping until all fluorescence was gone while the images were taken every 1 s with an exposure time of 33 ms. The measured image files were analyzed using a homemade MATLAB (Mathworks) program. The program first determined the center of a spot and then added up the intensity of the pixels around the center. We obtained the corrected fluorescence intensity by subtracting the background intensity we picked up a dark area close to the tip and calculated the total intensity for the same number of pixels. Following this method, we measured the trajectory of fluorescence intensity over time during continuous illumination of the labeled tip using a 405 nm laser for ~1500 s. The initial fluorescence intensity was 1943 ± 278 units. The trajectory of fluorescence intensity showed several steps (Fig. 4(b)), each with an intensity change of ~600 fluorescence units. Assuming that each step down in intensity was

caused by the bleaching of a single QD, we estimated that the tip started with three QDs. In replicate experiments, we consistently found fewer than 5 QD molecules on the tip using our method (data not shown). This result shows that we bind very few QDs on the tip.

To measure the number of molecules by another method, we compared the fluorescence intensity of the QDs on the tip to the fluorescence intensity of single QDs spread on a coverslip. We pipetted a dilute solution of QD525 on a coverslip, spun in a spin coater, and allowed the solvent to evaporate. We measured the fluorescence intensities of QDs on the coverslip and on the AFM tip using identical laser power and CCD camera settings. The fluorescence intensity of a single QD molecule was 1700 ± 600 units, while the QD on the tip showed an intensity of 1943 ± 278 units. This result suggests that a single QD molecule resided on the tip. Taken together, these results confirmed that our method of dipping the tip in paper labels the end of AFM tip with either single or very few molecules.

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